Atty. Dkt. No. 056859-0131

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Bangalore Eshwar Amita RANI et al.

Title: A PROCESS FOR THE PRODUCTION OF EGG YOLK ANTIBODIES FOR ORGANOCHLORINE PESTICIDES

Appl. No.: 09/973,199

Filing Date: 10/10/2001

Examiner: Phuong N. Huynh

Art Unit: 1644

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Declaration Under 37 C.F.R. § 1.132

1. I, Bangalore Eshwar Amita Rani, age 42 years, residing at INDIA, and a citizen of India, do hereby state as follows.

2. I am a Scientist at the Central Food Technological Research Institute, Mysore, India. I graduated in the year 1982 from Bangalore University located at Bangalore, Karnataka, INDIA. I completed my Master's Degree in 1984 from Bangalore University at Department of Zoology, Bangalore, Karnataka, INDIA. Subsequently, I completed my doctoral degree in Zoology from the University of Mysore, Karnataka, India in the year 1991.

3. After completing my doctoral degree, I took up my first assignment as a Lecturer in Zoology with the Department of Studies Zoology, Manasagangotri, University of Mysore, Mysore, Karnataka in the year 1992. After that, I joined the Food Protectants and Infestation Control Department, Central Food Technological Research

Institute, Mysore, India in year 1993. I am continuing to work with the institute for last 11 years and am presently working on Pesticide Residue Analysis Immunoassay and Toxicology.

4. Four conjugates were synthesized: DDT-OH conjugated to ovalbumin (OVA) and bovine serum albumin (BSA), DDA-GABA conjugated to OVA and BSA. DDT-OH-OVA conjugate gave the best antibodies in rabbits, while in the chicken DDT-OH-BSA gave good antibodies. This is indicative of the varied reaction to the same antigen depending on the animal system. This also demonstrates that rabbit HCH antibodies produced by Beasley can not form a basis for predicting chicken HCH antibodies.

5. The Trichlorobenzene hapten for the immunoassay of HCH synthesized by Beasley *et al.* was conjugated to OVA and keyhole limpet haemocyanin (KLH) and not to BSA for raising antibodies in rabbits. When trichlorobenzene containing a γ-aminobuyric acid spacer arm that means increase of a methylene group was synthesized (See Scheme 1, presented below), and rabbits were immunized with conjugates of this hapten (HCH-2), it was found that the antibodies recognized the hapten itself but not trichlorobenzene (the target molecule). (A. Pasha and Amita rani (2003), Unpublished work). Both HCH-1-HRP and HCH-2-HRP were employed as tracers but it did not help.

6. When HCH-1 hapten contained a β-alanine spacer arm was conjugated to BSA, it was found that this was most useful for raising antibodies in chicken. The sensitivity of the assay was higher than the published method in which rabbits were used to raise antibodies.

7. Endo-1I hapten for endosulfan was conjugated to OVA and BSA and antibodies were raised both in rabbits and chicken. Endo-1-OVA gave the best antibodies in rabbits whereas Endo-1-BSA was best to raise IgY antibodies. Endo-2 hapten protein conjugates did not give better antibodies and the Endo-diol-HRP conjugate was not useful as tracer.

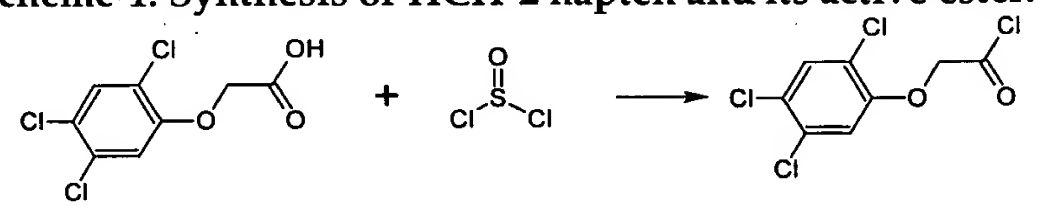
8. The titer of the antibody produced in chicken is at an average of 100mg /egg. (See Fig. 1. below). The eggs are collected for almost 3 months i.e. 90 eggs , 90x 100 mg = 9000mg or 9 g/ hen. Conservatively, this can be estimated to be an output of 5g/hen, which is almost 70 times more than the yield of the rabbit antibodies. This result would not have been expected based on the teachings of the prior art.

9. In the chicken the indirect ELISA worked best at 0.04 ug of the antigen and 0.25 ug of the antibody against 1/5k secondary – HRP conjugate dilution. (IC 50 = 10 ppb) (FIG.2). Fig3 shows the results of the competitive ELISA against 6 HRP – conjugates with an IC 50 value of 100 ppb. These data indicate that the chicken antibody is better than the rabbit antibody.

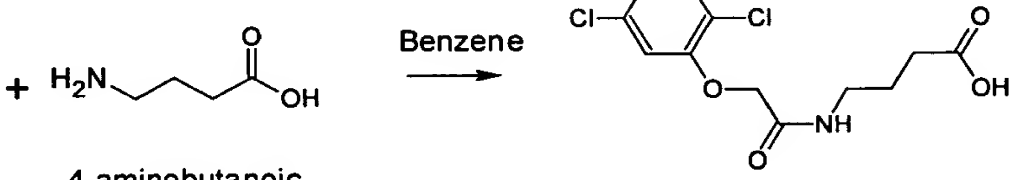
10. In the rabbits the competitive assays were most sensitive when DDA-GABA-HRP conjugate was used as tracer while DDT-OH-HRP did not give good response. (FIG.4) (IC 50 = 100 ppb).

11. Scheme 1

Scheme-1: Synthesis of HCH-2 hapten and its active ester:

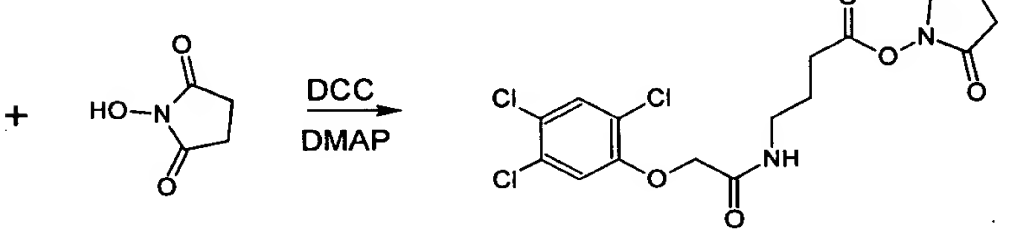


(2,4,5-trichlorophenoxy) acetic acid



4-aminobutanoic acid

4-{[(2,4,5-trichlorophenoxy)acetyl]amino}butanoic acid



N-{4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutyl}-2-(2,4,5-trichlorophenoxy)acetamide

12. Figures 1 – 4

Fig 1 : ESTIMATION OF ANTIBODY TITER

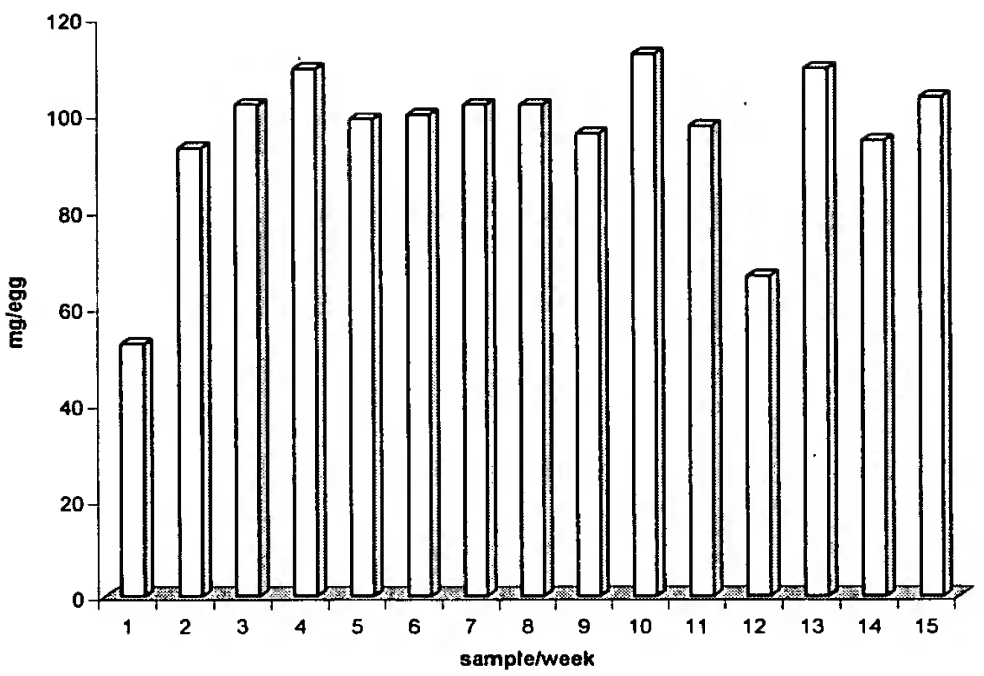
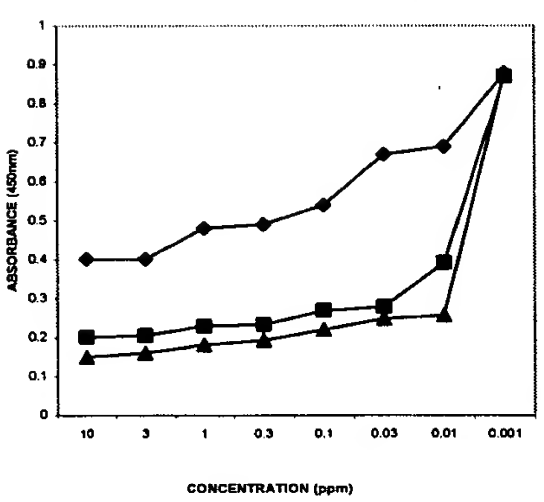
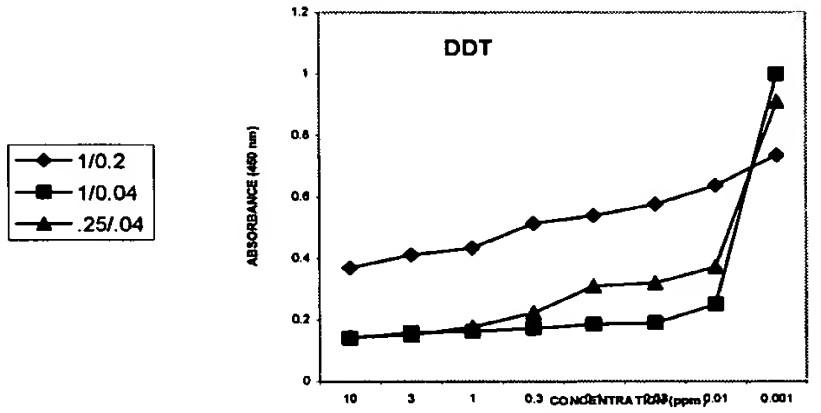


Fig 2: CHECKER BOARD ANALYSIS OF ANTIBODY AND ANTIGEN





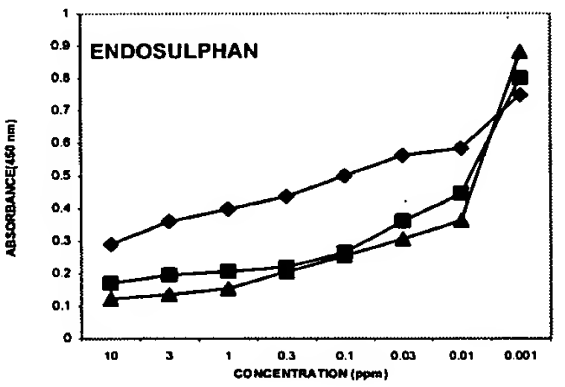


Fig 3: CHECKER BOARD ANALYSIS FOR DDT ASSAY WITH DIFFERENT ENZYME CONJUGATES

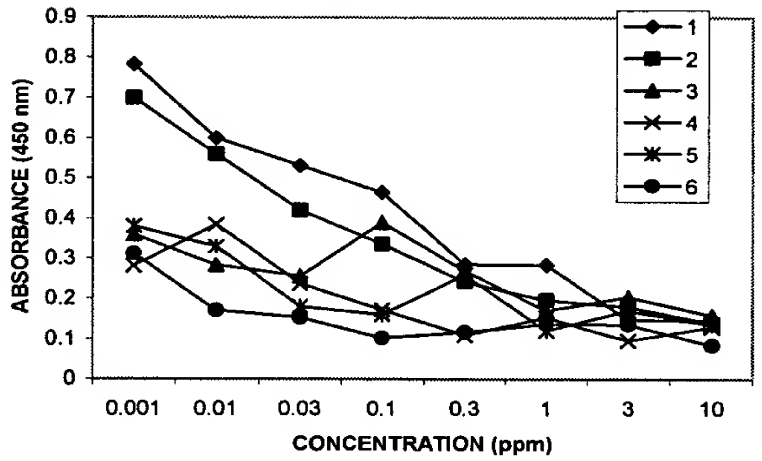
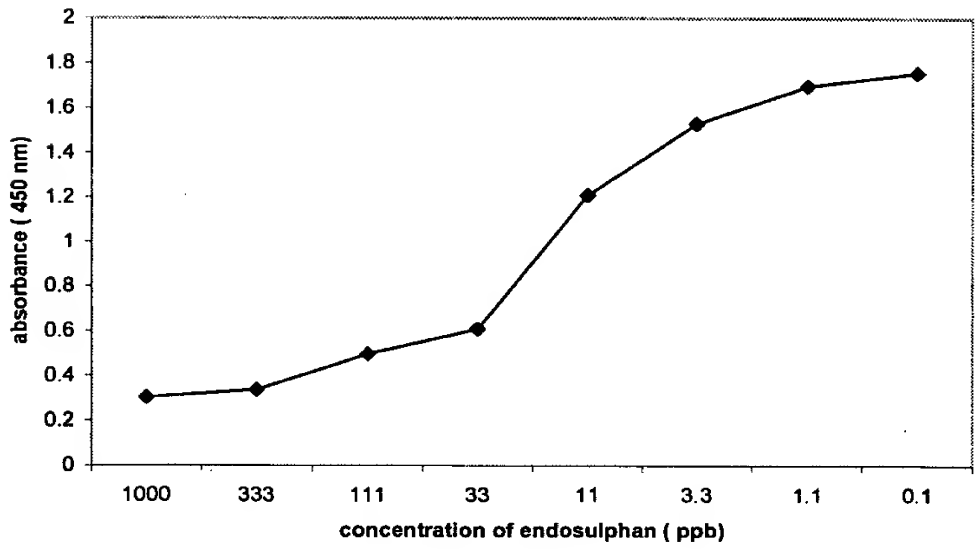


Fig 4 : Standard graph of DDT (rabbit antibodies)



13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and

further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Dated: 30th Sept '04

BANGALORE ESHWAR AMITA RANI